THE SEQUENTIAL DEPENDENCE OF in-Vitro FREEZE-DRYING AND IRRADIATION ON THE BIOMECHANICAL PROPERTIES OF RAT BONE.

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by

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THE SEQUENTIAL DEPENDENCE OF *IN-VITRO* FREEZE-DRYING AND IRRADIATION ON THE BIOMECHANICAL PROPERTIES OF RAT BONE. Robert Lawrence M. Randall (Sponsored by Richard R. Pelker, Gary E. Friedlaender). Department of Orthopaedics and Rehabilitation, Yale University, School of Medicine, New Haven, CT.

Sprague-Dawley rat femurs were subjected to *in-vitro* three megarad irradiation and/or freeze-drying to investigate whether these processes have an order dependent effect on the biomechanical properties of bone. Forty rats were randomly divided into 4 experimental groups of 10: 1) irradiated, 2) freeze-dried, 3) irradiated then freeze-dried, and 4) freeze-dried then irradiated. The femurs were harvested with the right designated as experimental while the left served as matched contralateral controls. Following the various treatments the bones were inspected for microfractures and then torsion tested. Data analysis within each group was performed using the paired t-test. The experimental values were also normalized against the respective contralateral controls as relative ratios (experimental/control). Intergroup differences were assessed for torsional strength and stiffness using the analysis of variance (ANOVA) of the relative ratios for each group. Microfractures were observed in nearly all (>85%) of the specimens that were subjected to freeze-drying as part or all of their treatment. The torque relative ratios demonstrated a statistical difference ($p<.05$) between group 1 (irradiated=1.0) and the latter three groups (freeze-dried=.32, irradiated then freeze-dried=.40 and freeze-dried then irradiated=.14). Differences between the 3 latter groups were not statistically significant. However, a trend was noticed. Bones that were freeze-dried then irradiated appeared weaker than those either freeze-dried alone or irradiated and then freeze-dried. The stiffness of the bones exhibited a similar pattern. The data suggests that a noticeable sequential dependency may exist but a significant order dependent effect could not be established by this study.

Foreword

I wish to gratefully acknowledge the guidance, support and constructive criticisms of Drs. Richard Pelker, Gary Friedlaender and Manohar Panjabi. My baccalaureate training having been in molecular biology I was a relative novice to the field of biomechanics when I began this exercise. Nevertheless Dr. Pelker was willing to sponsor and counsel me as I entered an unfamiliar realm. I am truly grateful for that as I have learned a great deal since. Working under the auspices of Dr. Friedlaender has been very inspiring as well. As a world renowned authority in bone allograft research he has kindled in me a professional and academic venue. Dr. Panjabi, an internationally recognized expert in biomechanics, has always made himself available to me when I have had questions although I regret not having taken advantage of his services more often. My future energies in research will return me to the molecular level however I truly appreciate the opportunity to have worked in the field of biomechanics with such distinguished scientists.

The technical assistance of Nancy Troiano and Stephanie Jacobson is gratefully appreciated. I also wish to thank Sarah Whitaker for her continued graphics assistance in presenting this material at the Orthopaedic Research Society, Yale Orthopaedic Association Post-Graduate Disputations, Yale Student Research Day , publication submission, and in this thesis.

The Department of Orthopaedics and Rehabilitation at the Yale University School of Medicine including its outstanding faculty, housestaff and terrific administrative assistants is thoroughly appreciated. I have known many of the department's members for the duration of my medical school education and remember sharing laughs as well as posturing myself in respectful intimidation in awe of their production of excellence.

The experiments as described in this thesis were undertaken by the author in the biomechanical laboratory in the Department of Orthopaedics and Rehabilitation at the Yale University School of Medicine. Data analysis was performed by the author with the assistance of Richard R. Pelker, M.D., Ph.D. Consultation was provided by Richard Pelker, M.D., Ph.D., Gary E. Friedlaender, M.D., and Manohar M. Panjabi, Ph.D.

Dr. Pelker is a recognized authority in the biomechanics of allografts. He has performed and published numerous experiments in the field to date. It is the techniques which he, Dr. Panjabi and others had developed previously that were utilized in this study. However, while this project complements data published in the past it is an independent investigation and not a step in a larger study.

Unfortunately, this thesis is unable to provide a definitive answer to the question asked. Nevertheless, I hope future energies on behalf of myself and others will be able to address this and related issues in the field of bone allografts.

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Introduction

Why Allografts?

Bone allograft transplantation has proven to have important clinical applications. While osseous autografts are the standard by which other alternatives are measured, they also have potential disadvantages. These include increased morbidity associated with the donor site, such as possible infection, significant hemorrhage, sacrifice of normal tissue, increased post-operative discomfort and cosmetic change. At times, available autograft is quantitatively and/or qualitatively insufficient for its intended biologic or biomechanical functions (37).

Sterile allograft bone may be obtained by using aseptic technique during graft retrieval and/or secondarily sterilized by treatment with megadose irradiation or ethylene oxide. The tissue can be preserved and stored by a number of techniques, most commonly deep-freezing or freeze-drying, until required for transplantation. Only the site to be grafted is disturbed during procedures using bone allografts and the quantity of available reconstructive tissue is far more abundant than from autogenous sources. However, allogeneic bone is associated with immunologic responses that probably impact upon their biology and function although the nature and magnitude of these changes remain unclear in humans (30). Nevertheless the need for increased sources of bone graft coupled with significant clinical success has encouraged and motivated investigative approaches designed to improve our understanding of and results with bone allografts (26).

Biomechanical Considerations in Allograft Technology

The biomechanics of bone used for allotransplantation have been studied extensively (10,11,25,34,42,54,56,57,65,72). Structural integrity of the graft is important especially when the host site is involved in load bearing. In one series (43), 16.5% of bone allograft cases resulted in fracturing of the graft. In evaluating the biomechanical properties of allografts several factors that may affect the graft's physical structure must be considered. The initial properties of the graft at the time of harvest, the effects of the various preservation, storage and sterilization procedures, and the biomechanical effects of biological incorporation and remodelling of the graft all influence the physical quality of the graft. Since bone allografts may experience several different mechanisms of loading, including compressive, torsional, bending, shear or tensile, alterations in each of these failure modes following various preservation techniques are of clinical importance (54).

The material quality of the donor graft is determined in large by the original properties of the bone at the time of harvest. These properties are influenced by numerous factors including age, sex, physical constitution and health of the donor, the anatomic site from which the bone was taken, the type of graft (cortical versus cancellous) and the geometry of the graft. This last factor, the graft's size and shape, may be the most important variable in determining the mechanical strength of the graft. The cube of the cross-sectional diameter or width of the graft is directly proportional to its strength. Thus using a larger piece of weaker bone graft can compensate for the intrinsic potential of the material to fail given that the host site can accommodate a larger graft (55).

The load experienced by the allograft also influences the fatigability of the bone. In humans, bone tends to be twice as strong in compression (137-196 MPa) as in tension (88-108 MPa) (23). Accordingly, bending or torsional loading

at a given stress level is more deleterious than longitudinal (osteonal) loading. Loading at a slower rate will more likely result in failure than if loaded quickly (55).

Bone is generally strongest between the ages of 20 and 39 in humans reflecting the period of greatest bone mineral content (54). While it gradually decreases thereafter people in their seventies will retain 70-85% of their maximum strength.

Operative technique affects the strength of the graft as well. Fixation of the construct causes shielding of the loads experienced by the graft. Because incorporation and remodelling of the allogeneic tissue is a relatively slow process the fixation device, whatever used, must protect the graft for an adequate length of time from the excessive loading potentially experienced by the graft. The shielding must be selective for the loading types vary as a function of the anatomic site. In addition, bone graft transplanted into a mechanical environment where it is subjected to absolutely no physical loading will tend to resorb (55).

The ultimate determinant of whether a graft will mechanically fail depends on the biology and immunology of the graft-host interface. Until the graft is incorporated and vascularized, the graft cannot remodel and accordingly is vulnerable to fatigue (low repetitive loading) as well as traumatic (massive single loading) failure. As in fracture healing several stages of repair occur. There is an initial period of low strength and stiffness resulting from early bony resorption and decreased bone density. With healing and remodelling new bone is deposited and there is an increase in the strength and stiffness until the site approaches its pre-fracture state. A similar process has been described for allografts (58). The rate at which this process occurs is itself dependent upon several of the above mentioned factors such as allogenicity, patient's age and state of health, adjuvant patient treatments (chemotherapy and radiation

therapy), the mechanical environment and sterilization and preservation techniques.

Radiation and freeze-drying have been used extensively as methods for sterilization and preservation of bone allografts. Irradiation, at megadose levels, is required to effectively sterilize bone (18). Ethylene oxide is also an effective sterilant but its toxic by-products must be adequately removed prior to human transplantation (38,53). In addition bone sterilized using this method is weaker in compression as compared to untreated bone (62). While the optimal irradiation dosage for sterilization remains unclear most banks using this approach use 2.5 to 3.0 Mrads (7,32,45,69,71). These doses surpass the 2.0 Mrads considered to be effective in destroying the majority of bacteria and viruses residing in human tissues. The level of irradiation required for inactivation of HIV within osseous tissue remains unknown but appears much higher than those previously suggested based upon *in vitro* studies of the virus in suspension (19,29). Irradiation dosages greater than 3.0 Mrads have been shown to alter the biomechanical properties of bone (56,72). Some investigators, however, suggest that increasing the dosage level above 3.0 Mrads will not significantly effect the material properties but may provide adequate inactivation of HIV (47).

Preservation is a necessary consideration so as to have suitable bone available on short notice. Freezing of bone to -20°C will have little if any consequences in terms of the physical nature of the graft (65). Yet, at this temperature enzymatic degradation is not entirely halted (54). As a result, it is routine to preserve grafts by freeze-drying or freezing to colder temperatures (-70 to -80°C) or in liquid nitrogen (-196°C). Deep freezing has not caused any deterioration of mechanical properties of bone, regardless of storage temperatures (57).

Freeze-drying has been used as a method for the preservation of bone for approximately 40 years (24). This approach allows storage of tissues at room temperature for an extended period of time. While an effective preservation technique, freeze-drying is also known to diminish the biomechanical parameters of bone (11,42,57,72). Despite the physical restrictions of freeze-dried grafts, as discussed in the Literature Review below, they have been used for many years with clinical success (64,68).

Goal

The treatment modalities of freeze-drying and irradiation have specific independent effects on particular parameters of the osseous tissue. For example, freeze-drying has been shown to have a more deleterious effect on torsional strength than compression (57). In addition the treatments may effect the material in concert. Studies have shown that the combination of irradiation and freeze-drying can have an additive effect on a particular mechanical parameter of bone (11,34,72). Bright and Burstein reported that bone exhibited no change in compressive strength when first freeze-dried or irradiated with 3.5 Mrads but was significantly weakened when irradiated and subsequently freeze-dried. Investigations with soft tissue indicate that when tendon is freeze-dried and irradiated, biomechanical properties differ depending upon the order in which these two processes are carried out (28). In essence there appears to be a sequential dependency of these treatment modalities reflected in the mechanical properties of the tissue. Since mechanical failure remains an important clinical issue for bone allografts following implantation, it is of significance to define whether the order in which the bone is freeze-dried and irradiated has a similar sequentially dependent effect on the biomechanical strength of the osseous graft. This study was designed to address that question.

Review of the Literature

BIOMECHANICS

An understanding of material properties and certain biomechanical concepts is necessary to analyze the results of this investigation.

The principles applied in this study pertain to viscoelastic materials. Viscoelasticity refers to the rate-dependent behavior of a material to loading. It involves two components: viscosity and elasticity.

Elastic Deformation and Elastic Modulus

A solid can be defined as any substance that responds *elastically* to a *stress*. If a substance is subjected to a given force it will deform as a function of the material substance, the force and the initial dimension of the substance. In discussing elastic deformation the terms *stress* and *strain* must be defined. Stress is equal to a given force applied over a given area.

$$\text{Stress} = \text{force}/\text{area}$$

Strain is the deformation of a material relative to its initial dimension.

$$\text{Strain} = \text{change in dimension}/\text{initial dimension}$$

The unit of measurement for stress is the *pascal* (Pa) or Newton per square meter. Strain has no unit of measurement.

For a given material, whatever the shape, if the deformation is elastic, then the following relationship exists:

$$\text{Stress} = \text{constant} \times \text{strain}$$

The constant above is the *elastic modulus* and the relationship is referred to as *Hooke's law* . The elastic modulus depends on the type of deformation (e.g. tensile, compression, shear, torsional) and on the substance but not on the

geometry of the sample. The elastic modulus is an intrinsic property of a given material.

The linear relationship between stress and strain holds over a limited range. If the stress exceeds the elastic limit the material deforms *plastically*. Plastic deformation results in the sample not resuming its initial shape when the applied stress is removed. In this state the intrinsic property of a given material is defined by the *plastic modulus*. If the force increases further the material will *fracture*.

The above material relationship can be plotted as a stress-strain curve (Fig. 1).

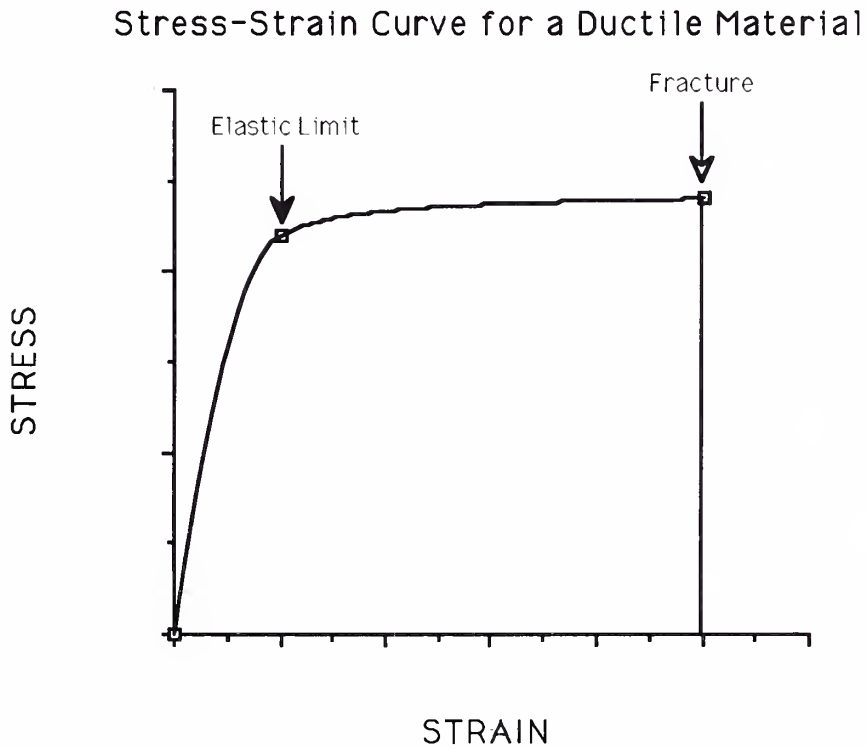


Fig. 1. A typical stress-strain relationship for a ductile material. The linear slope of the curve defines the elastic modulus until it approaches the elastic limit at which point the slope then becomes exponential. After passing the elastic limit the slope then defines the plastic modulus until fracture. Ductile materials can sustain considerable plastic deformation (between the elastic limit and the fracture point). For relatively brittle materials such as bone, the fracture point and elastic limit are very close (see below).

Load-deformation curves represent the *structural* or three dimensional properties of a substance including material, shape and size. These properties provide a material with *stiffness*. Stiffness is similar to elasticity in that it is a resistive quality to deformation but it is distinct in that it incorporates the distribution of the material in space. This is clarified by an example by White and Panjabi (77). The elastic modulus of stainless steel is greater than that of bone. However a steel hip screw is relatively less stiff than a human femoral neck. While this may seem counter-intuitive it is explained by the analysis of the amount and distribution of the two materials. The screw with a smaller radius has its material closer to its axis. The neck of the femur with a greater radius has its material distributed over a greater distance from the axis instilling more resistance to bending through the greater moment of inertia of its cross-sectional area (Fig. 2).

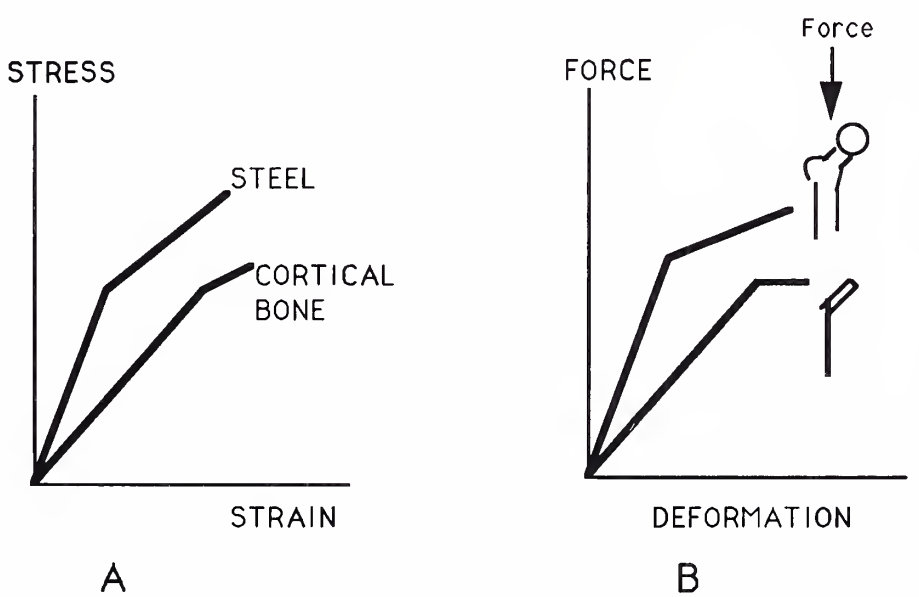


Fig. 2. A) Steel has a greater elastic modulus than cortical bone. B) However the stiffness of the femoral neck is greater than a hip screw secondary to the advantageous distribution of material in the femoral neck (77).

Viscosity

Viscosity is the property of a material enabling it to resist shear stress. A shear stress is a force applied to the surface of a material in a parallel plane. The coefficient of viscosity, η, is defined as

$$\eta = F/A \,/_{v\,/l}$$

where F/A is the shear stress needed to overcome the material resistance and *l* is the distance beween adjacent planes within the material. *v* is the relative velocity, or dispacement per unit time, of the material in the plane experiencing the load as compared to the adjacent material plane. By this formula one can note that the viscosity of a material is time or rate-dependent. With a greater velocity of loading the viscosity decreases. Thus in the load-dispacement curve previously described, a greater strain rate will cause the slope of the curve to become steeper. This steeper slope will produce a greater failure load and energy absorbed to failure so long as the displacements are similar. In this study the strain rate was a constant.

Torsion

This study investigated the effect of torsion on the biomechanical strength of bone. Torque is a rotational force exerted on a body applied tangentially to the body at a given distance away from the center of mass (CM) of the body. The action causing the rotation is a function of the magnitude and direction of the applied force and on the point of application (Fig. 3). For example, when entering a revolving door one applies one's outside hand knowing that a much greater force would be necessary to turn the door if the inside hand near the axis of rotation were used instead. Thus torque may be defined as,

τ = **force x moment arm**

The unit is the newton meter (Nm).

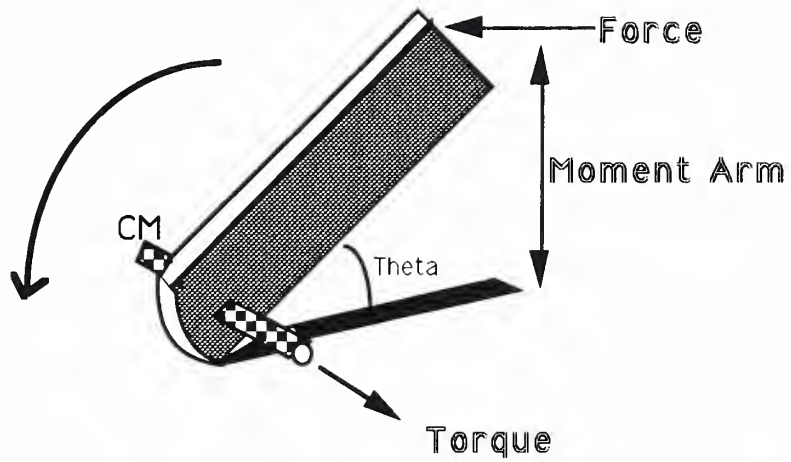


Fig. 3. Torque is the result of a force acting on a given body at a distance (moment arm) from the center of mass (CM) of the body. The direction of the torque is perpendicular to the direction of the force applied.

In the biomechanics of bone, torsion refers to the application of torque. The torque is defined as a couple of equal, opposite and parallel forces separated by a distance (Fig 4). In this study the torque exerted was a constant provided by the torsion testing device with a counter (opposite and parallel) torque provided by the mounted bone. The torsional strength of the bone was considered to be the peak of the load-deformation curve as it was subjected to a torsional load.

Stiffness

Stiffness is the resistance of a material to a displacement or deformation. In terms of torsional stiffness it is the number of radians the structure is deformed by a given torque up to failure and is given by the slope of the load-deformation curve.

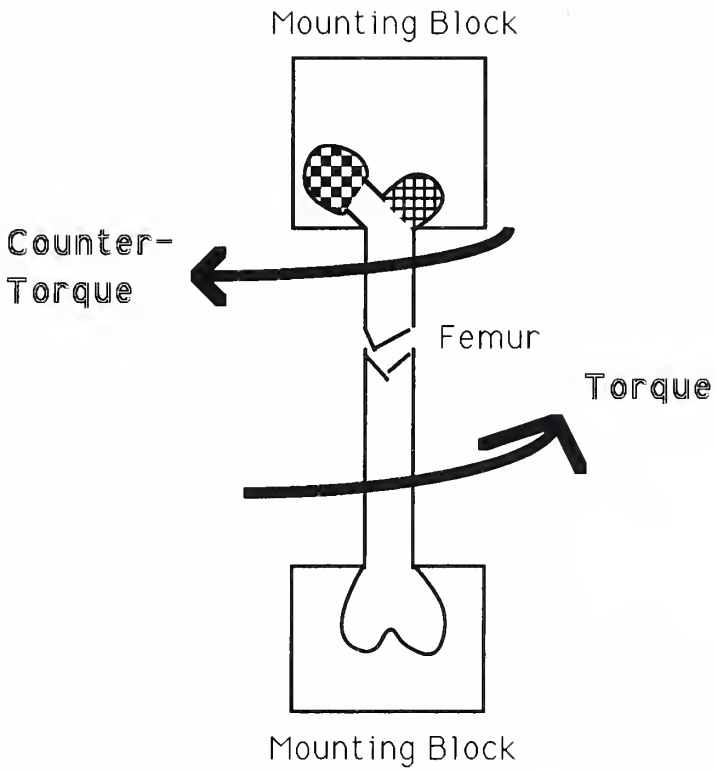


Fig.4. In biomechanics, torsion refers to the coupling of forces in parallel yet opposite directions with respect to the long axis of the bone. Experimentally the bone is mounted in blocks which is then placed firmly within a torsion testing device (see Materials and Methods section) and a constant rotational force is exerted. The peak of the load deformation curve is the torsional strength.

Radians

One radian is defined as the angle subtended by the arc whose length equals the radius of the circle (Fig. 5). The angle, θ (Theta), measured in radians is given by the ratio of arc length to radius,

$$\theta = \text{arc length} / \text{radius} = S / r$$

Since the arc length of a circle of radius r is $2\pi r$, the conversion between radians and degrees is given by the condition 2π radians = 360°. Because the angle θ is defined by a ratio of two lengths, angle is dimensionless.

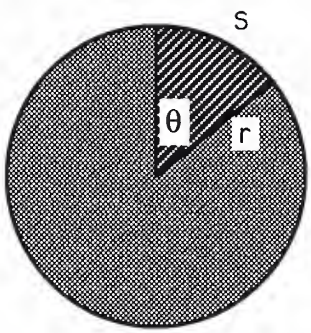


Fig. 5

Angular Deformation at Failure

This was measured in degrees and refers to the number of degrees the bone was rotationally deformed at the point of failure.

Energy absorbed at failure

Energy is defined as the capacity to do work. Work is performed by a force (torque) acting through a distance (angle). This was determined by integrating the area under the load-displacement curves (Nm*rad).

$$E = \int T d\theta$$

where T is the torque and θ is the torsional deformation or angle. Note that the units for energy (Nm or Joules) are the same as those for the torque because the angle has no units.

BONE AS A MATERIAL

Bone is a solid with a stress-strain relationship similar to many hard materials (Fig. 6). As stated above the elastic modulus of a material is dependent upon the type of deformation the material experiences as well as the

intrinsic properties of the material. The state of the bone (e.g. dry vs. wet) will effect the relationship as well (Fig. 6). Therefore when bone is tested in various types of loading, different ultimate strength values will result depending upon the type of load, the specific microstructure of the bone that is tested, and the state of the bone (27).

The gross anatomy of bone reveals that it is not a homogenous material. A mature long bone consists of the diaphysis, the expansive metaphysis and the epiphysis. The diaphysis is mostly dense cortex which is thick throughout but tapers at the metaphysis to become a thin shell. The shaft encloses the hollowed medullary cavity which contains some trabecular bone. The metaphysis and epiphysis are primarily trabecular bone.

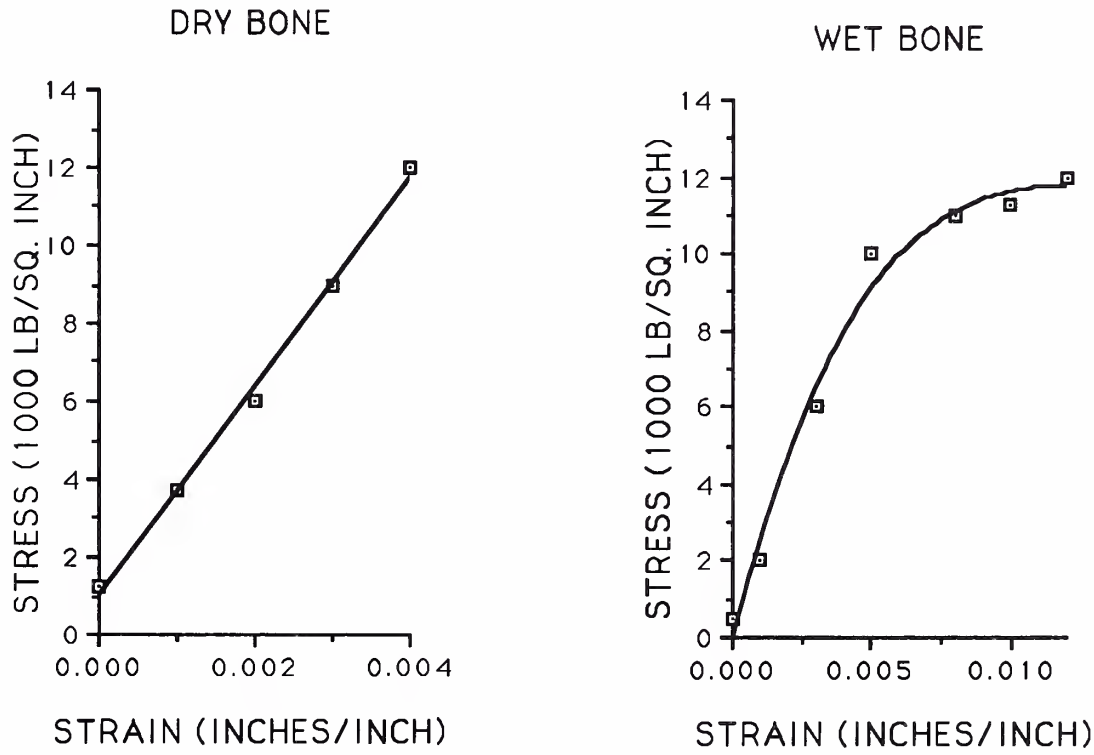


Fig. 6. Stress-strain curves of human femoral bone. Note how the relationship is affected by the state of the bone (23).

The basic structure of cortical or compact bone is centered upon the osteon or Haversian system. Bony columns of lamellae circumscribe neurovascular Haversian canals. The columns are arranged along the lines of stress exerted on the bone (Wolff's Law). Volkmann's canals run more or less perpendicular to the Haversian system so as to permit communication with the endosteum and periosteum. The construct has a high bone density. Trabecular bone on the other hand consists of a network of fine, irregular plates giving the bone a spongy appearance and a lower bone density.

Microanatomically bone is a nonhomogeneous anisotropic composite material. It is a specialized form of connective tissue in which the extracellular components are mineralized, endowing the osseous material with substantial rigidity and strength while still retaining a degree of elasticity. Approximately two-thirds of the weight of dry bone is inorganic hydroxyapatite, $3Ca_3(PO4)_2 \cdot Ca(OH)_2$ in the form of crystals roughly 200 Å long with an average cross-section of 2500 $Å^2$ (9). The remainder of the material is organic collagen, predominantly Type I, with the hydroxyapatite arranged along its length. The arrangement of the collagen fibers differs depending upon the type of bone. In woven bone it is tangled while in mature bone it is organized into lamellae.

Bone is therefore a composition of collagen and hydroxyapatite. Apatite is strong and stiff with a Young's modulus (elastic modulus for tensile stress) of 16.5×10^{10} Pa (steel = 20×10^{10} Pa, aluminum=7.0×10^{10} Pa). The modulus for collagen equals $.124 \times 10^{10}$ Pa. The modulus for bone is intermediate between its two components with a value of 1.8×10^{10} Pa. However, because of its composite nature the strength of bone is actually greater than apatite or collagen as the collagen prevents the stiff apatite from cracking while the apatite prevents the soft collagen from yielding (27).

A mathematical model for the strength of bone would prove beneficial yet has not been developed secondary to the complexities of such a formula. Aside from the specific mechanical properties such as density, Young's modulus, shear modulus, plastic modulus, viscoelastic properties and stress and strain at failure, the microstructure of the segment of particular bone must be closely analyzed for trabecular pattern and percent cortical versus spongy bone. To further complicate calculations it has been demonstrated that the correlation coefficient of bony strength and bony density is merely 0.40-0.42 (2,3,4,63). Because of these factors much of the work in the field of bone biomechanics and the effects of treatment modalities has been empirically based.

Irradiation as a Sterilant

While some tissue procurement cannot be done under sterile conditions, at other times tissues to be transplanted become inadvertently contaminated during harvest or storage. Over the years, irradiation sterilization has become one of the most widely used methods of decontaminating human tissue. This section will discuss the various potential types of irradiation as well as some of the considerations involved with this modality.

In considering irradiation sterilization one must consider numerous conditions that affect the physics and biology of the tissue graft as well as the technical process. These conditions include: 1) the type of irradiation employed; 2) the dosage delivered to obtain complete and reliable sterilization; 3) the type of bacteriologic or viral contamination present; 4) the type, size and condition of the tissue including its geometry, texture, hydration, temperature and storage state, whether in vacuum or air; 5) the package conditions; 6) the potential development of tissue activation and 7) the biologic and biomechanical effects (74). This latter consideration will be reviewed in a subsequent section.

There are at least six types of irradiation that could be considered for sterilizing human tissue (Table 1). X rays provide adequate sterilization although the required exposure time is much too long. Gamma rays however, are the most commonly used source and have excellent tissue penetration. Exposure time may need be up to 24 hours depending upon the dose. If cobalt 60 is used, as in this study, the rate of delivery is increased and thereby the time can be decreased. Of note however is that with increased rate of delivery there is increased tissue heating.

Accelerated electrons produce gamma rays within the tissue by Compton scattering when they collide with tissue molecules. Electrons can achieve tissue sterilization within an hour (73). However because of their negative charge electrons have diminished depth of penetration as they are easily deflected and slowed. Neutrons can be used for sterilization but cause a very high degree of tissue activation and are of no clinical value. Protons and α-particles have poor tissue penetration and are not used clinically.

Table 1. *Irradiation Types and Their Limitations* (12)

Source	Exposure Time	Penetration	Activation	Availability
X ray	1 year	Very good	None	None
Gamma rays	<1 year	Excellent	None	^{60}Co source
Electrons	<1 hour	5 cm	Some	Lin. Accel.
Neutrons	Weeks	Good	V.Large	Nucl.React.
Protons	Weeks	Poor	Large	Cyclotron
α-partics.	Weeks	Poor	Moderate	Cyclotron

The two principal sources of irradiation that have been used most often are gamma rays and accelerated electrons. The latter is used primarily with skin grafts. Gamma rays from a cobalt 60 source are neutral particles and are not deflected. They penetrate osseous tissue well and are limited only by the size of the source and the length of time acceptable as tissue heating can be a significant problem.

Of particular interest is the mechanism by which irradiation produces sterilization. Gamma rays produce ionized particles. These secondary particles split other molecules including water. Water is split into hydrogen and hydroxyl-free radicals. The irradiation also directly damages the nucleic acid and breaks down crosslinks in the tertiary configuration of proteins (74).

The physical state of the tissue to be irradiated therefore affects its susceptibility to sterilization. In the freeze-dried state water molecules are relatively scarce. Thus for a given dosage of irradiation less hydroxyl-free radicals are produced per gram tissue. Therefore a larger dosage of irradiation may be necessary to produce effective sterilization in freeze-dried tissue (20).

Freeze-Drying of Bone

A great deal of clinical and experimental work has been done over the past few decades regarding freeze-drying of bone. This section will define the process and discuss the importance in establishing and following a protocol. Relevant experimental biomechanics will be reviewed in the subsequent section.

The process of freeze-drying involves removing water from frozen material by sublimation in a vacuum. Thus crystalline water is lyophilized to vapor directly bypassing the liquid state. The vapor is then converted to crystals on a condenser that is substantially cooler than the material being freeze-dried.

Water can be divided into two categories: 1) free water and 2) water bound to macromolecules or structured water. Free water is the basic component of biologic fluids maintaining the minerals, sugars, amino acids, lipids and proteins in solution. When cooled sufficiently it crystalizes into ice. Bound water on the other hand, while small amounts may be mildly affected by physical and thermal changes in the environment, cannot change its state without causing a profound chemical and physical alteration in the macromolecule.

Freeze-drying has been subdivided into two sequential phases (61). During the the first or primary dessication phase, water in the form of ice is removed. This phase is visually defined by the absence of visible ice particles. The second phase involves the extraction of bound or structured water and is demonstrable only indirectly by changes in the physicochemical composition of the material. The latter phase is much more slow relative to the first but does occur as the freeze-drying process continues. As the water content approaches absolute zero, which is experimentally difficult, frank morphologic alterations of the tissue structure are likely to occur. The alterations produced by freeze-drying in biological tissues are significant. Eukaryotic cells are virtually killed by the process while some microorganisms do survive (41).

The water that is not removed from the tissue by freeze-drying is termed the "residual moisture" or "residual water." The residual moisture levels are determined to quantitate the efficacy of the process. The most frequently used methods, based upon labor and financial restraints, are indirect and can be subject to variation. It is an important measurement nonetheless as the amount of water remaining in the tissue has a direct bearing on the characteristics of the tissue. Thus it is important to be regimented about protocols if one is to correlate structural characteristics with water content.

Regardless of the total length of the process a certain amount of bound water will remain in the tissue. However, the greater the amount of water remaining the greater sensitivity of the freeze-dried bone to environmental conditions.

There are several methods for determining the residual moisture of a freeze-dried material. The gravimetric method is the one utilized in this study and is reviewed in the Materials and Methods section. It is the oldest, simplest and the most popular. It is based upon the assumption that in a freeze-dried product there is a certain quantity of water for a given mass of the substance. If a sample is placed in a heating oven with a drying agent such as silica gels (P_2O_5) and heated to a given temperature and weighed intermittently, it will eventually reach a constant weight. This is defined in the formula,

$$R\ H_2O\% = {}^{m}/_{M} \times 100$$

"*R* H_2O" is the residual moisture, "m" represents the weight decrease due to water loss and "M" is the original weight of the object prior to heating. In many countries this is the official manner in which residual moisture is determined and its is widely used by the international scientific community. However, it is based on the false assumption that when the weight of the sample becomes constant it no longer contains water. In addition, a temperature must be selected that will be sufficient to drive off the residual water.

The Karl Fischer method employs iodine in a mixture of methyl alcohol and anhydrous pyridine supplemented with sulfurous anhydryde. Iodine, which is inactive in the anhydrous solution, oxidizes SO_2 to SO_3 in the presence of water while being reduced to hydroiodic acid which fixes the pyridine. The reagent initially a dark brown, fades in color. While a simple procedure it is difficult to standardize.

NMR is the gold standard of water determination. Since every proton of every water molecule in the specimen is reactive, this technique will provide an absolute measurement. This resource was not available at the time of this study.

EXPERIMENTAL HISTORY

Limb transplantation has been incorporated into mythology and lore throughout the history of mankind and has been depicted in religious art work for many centuries (6). Scientifically the study of bone utilization and potentiation was undertaken in the early and middle part of the nineteenth century as described by Ollier (48). In 1881 Macewen pioneered bone allograft transplantation with the reconstruction of the diaphysis of the humerus in a young child using a cadaveric specimen (40). Cadaveric sources were tried in other cases requiring segmental replacement of portions of the skeleton secondary to trauma, tumor resection and various other skeletal diseases. Further work by Barth (5), Phemister (59), Lexer (39) and Albee (1) supported the concept that such a technology was practical and by 1925 the field of bone allograft technology had been established.

Bone banking was initiated in the period of the poliomylelitis epidemic by such individuals as Inclan (33), Wilson (78), Bush (15),Kreuz et al (36), and Hyatt and Butler (31). Developments in the field lead to various modalities of bone processing such that after World War II the Navy instituted a large bone harvesting program. The first attempt at a methodologic preservation of cadaveric bone by freeze-drying was made by the Navy Tissue Bank in 1952 (24, 36). Bone was freeze-dried in order to store the osseous material in large quantities for utilization in the treatment of severe war wounds. Subsequently this technology was adapted for use in the civilian population and large, regional

tissue-banks were put into operation in the United States, the former Soviet Union, Eastern Europe, and the United Kingdom with the employment of deep-freezing and freeze-drying for long-term preservation (66). Initially, orthopaedic management included arthrodesis among other things, and hospitals "banked" small portions of bones in refrigerators and freezers. The surgeon would use these bone bits to supplement a fusion mass or as implant in cyst cavities or after curettage of a tumor. Innovative limb-salvaging surgical techniques were developed by Parrish in the United States (51,52), Ottolenghi in Argentina (49), Volkov in the former Soviet Union (76), Koskinen in Finland (35), and Mankin and associates in Boston (42,44) adding additional impetus for continued development of osteochondral banking technology.

Much of the scientific study of allografting was supported by the Navy Tissue Bank. Of particular interest was determining the effects of the various preservation techniques on the immunogenicity and biomechanics of the osseous tissue. Bonfiglio (8), Burwell (14), Chalmers (16) and Curtiss and Herndon (21), through careful animal experimentation discovered that frozen and freeze-dried intercalary or osteoarticulary allogeneic specimens had decreased antigenicity relative to fresh bone further supporting the clinical relevance of these modalities. Frankel in 1960 (25) was one of the first to evaluate the effects of freezing on the biomechanics of bone. Bending "breaking strength" was not significantly changed in cadaveric femoral necks stored at -25°C for several weeks as compared to fresh specimens. This work was supported by Sedlin in 1965 (65) using specimens machined into 2 X 1 and 2 X 2 mm. cross-sectional beams. After the bone was frozen to -20°C for three to four weeks they were thawed to 37°C and subsequently bent to failure. No significant effects were noticed as compared to fresh controls. Komender in 1976 (34) stated that deep frozen machined femora (-78°C) showed no change

relative to fresh controls in terms of torsional strength but did demonstrate a 10% decrease in bending compression.

In the late 1970's and early 1980's investigators began to look at the effects of freeze-drying and irradiation on bone strength. Bright, Burchardt and Burstein (10,11), using machined cadaveric tibiae and femora, conducted tension and compression loading tests. Bone was freeze-dried to a residual moisture content of less than 5% (please refer to Materials and Methods section for description of this technique) and then rehydrated prior to testing. After one hour of rehydration the elastic modulus returned to normal yet the plastic modulus remained elevated after 24 hours. They reported no change with regards to compression strength relative to frozen controls. In irradiated specimens only the plastic modulus was elevated as compared to nonirradiated samples. Specimens that were irradiated and freeze-dried were noted to be substantially inferior in terms of compression strength.

Triantafyllou et al. (72) performed three-point bending on machined adult calf bones. Specimens were frozen at -35°C for three days and then either freeze-dried and/or irradiated with 3.0-4.0 Mrads. The samples were rehydrated for two hours prior to testing. Controls were stored at -35°C. Their results revealed that the strength of freeze-dried bones was decreased to 55-90% of controls, the strength of specimens that were irradiated was diminished to 50-75% of controls and the combination of freeze-drying and irradiation lead to a decrease to 10-30% of controls.

Further work by Komender (34) using machined femora evaluated the effects of freeze-drying and irradiation on compression, torsion and three-point bending. The bones were either frozen to -78°C, irradiated fresh or freeze-dried and irradiated. The results were compared to fresh controls. Freezing alone had no effect on any of the biomechanical parameters. Irradiation with 3.0

Mrads resulted in a 10% decrease in torsional and bending strength but did not effect compression. At 6.0 Mrads torsion and bending strength were reduced by a total of 35% and 30% respectively while compression was also decreased by 20%. Irradiation of freeze-dried bone with 3.0 Mrads resulted in a 30% decrease in torsion, a 20% decrease in bending and no change in compression.

Work by Pelker and colleagues (56,57) revealed that different preservation techniques may effect specific biomechanical parameters differentially. Deep freezing of bone does not significantly alter the torsional or compressive strength of bone. Freeze-drying however, results in a substantial decrease in torsional strength but does not effect compression.

Haut et al. (28) in 1989 investigated the order of irradiation and lyophilization on the strength of patellar tendon. They noted a 25% decrease in the strength of the soft tissue following irradiation at 2.0 Mrads. Secondary lyophilization did not alter the strength of the tendon. If the tendon was freeze-dried prior to irradiation a 75% decrease in strength was noted. The material properties of soft tissue are different by definition than those of bone. Accordingly they may and do behave in a qualitatively similar yet quantitatively different manner when subjected to the treatment modalities of irradiation and freeze-drying. For example, in this case only an irradiation dose of 2.0 Mrads is required to reduce the strength of the tendon by 25%.

The experimental investigation of freezing, freeze-drying and irradiation on bone is summarized in the table on the next page adapted from Pelker (Table 2). The results of this study are included.

Table 2. *Effects of Preservation on Bone Strength* (55)

Investigator	Preservation Method	Bone Strength(% of Control) Compression	Torsion	Bending
Frankel (25)	Freezing(-25°C)*	---	---	100%
Sedlin (65)	Freezing(-20°C)*	---	---	100%
Komander (34)	Freezing(-78°C)	90%	100%	90%
	Radiation (1Mrad)	100%	90%	100%
	Radiation (3Mrads)	100%	90%	90%
	Radiation (6Mrads)	80%	65%	70%
	Radiation (3Mrads) & Freeze-drying	100%	70%	80%
Bright & Burstein (10,11)	Freeze-drying	100%	---	---
	Radiation (3.5 Mrads)	100%	---	---
	Radiation (3.5 Mrads) & Freeze-drying	Significantly Decreased	---	---
Triantafyllou et al (72)	Freeze-drying	---	---	55-90%
	Radiation (3-4 Mrads)	---	---	50-75%
	Radiation (3-4 Mrads) & Freeze-drying	---	---	10-30%
Pelker et al (56,57)	Freezing (-20°C)*	120%	100%	---
	Freezing (-70°C)*	122%	100%	---
	Liquid Nitrogen*	114%	100%	---
	Freeze-Drying*	120%	39%	---
Randall et al (60)	Radiation	---	100%	---
	Freeze-drying	---	32%	---
	Radiation (3 Mrads) & Freeze-drying	---	40%	---
	Freeze-drying & Radiation (3 Mrads)	---	14%	---

* Fresh controls (all others are frozen).

Materials and Methods

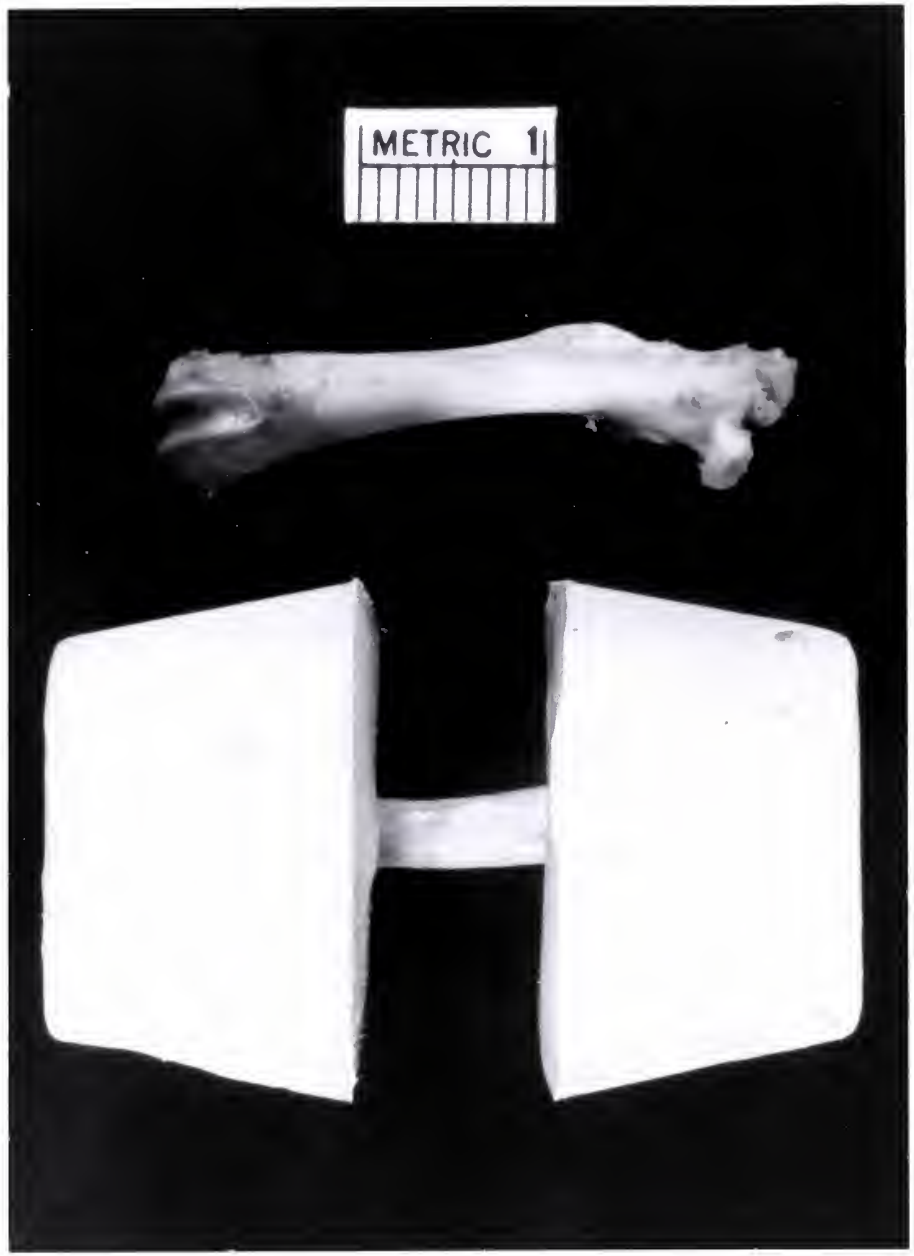
Femurs from adult female Sprague-Dawley rats weighing 275 ± 5 g were utilized in this study. Forty rats were randomly divided into 4 groups of 10. At sacrifice the right femurs were designated as experimental while the left served as matched contralateral controls. The right femurs from Group 1 were irradiated with 3.0 Mrads; Group 2 was freeze-dried; Group 3 was irradiated with 3.0 Mrads then freeze-dried; Group 4 was freeze dried and then irradiated with 3.0 Mrads. All bones were stored at -20°C for at least two weeks until they were tested.

The freeze-drying protocol involved the freezing of the bones to -70°C for 72 hours with subsequent lyophilization to a residual moisture content of 3% or less using a gravimetric assay. The freeze-dried femurs were weighed immediately following lyophilization and then subjected to 100°C until a constant weight was reached. This invariably required less than one hour. The heating served to dehydrate the tissue as much as experimentally possible, removing any residual moisture. This accounts for the slight loss in weight of each bone after heating. The residual moisture content following freeze-drying was approximated by the ratio of the weight of the femur immediately after freeze-drying to the weight upon removal from the oven (41). Bones were then kept at -20°C until mounting or irradiating, as determined by the experimental protocol.

A dosage of 3.0 megarads ±5% (2.87-3.14 Mrads) was administered at a rate of 4.9 Krads/min. to the 3 designated groups via gamma irradiation from a cobalt 60 source at room temperature (Isomedix, Mortin Grove, IL). Upon completion the bones were stored at -20°C. Group 3 underwent freeze-drying after irradiation while Group 4 was freeze-dried before irradiation.

Both ends of each bone were mounted in a quick setting dental casting material ("Die Keen Green", Columbus Dental, Miles Inc. St. Louis, MI) (Fig. 7).

Fig. 7. Rat femurs magnified 2X. On the left, a femur immediately after harvesting with most of the soft tissues removed. On the right, a mounted femur. The potting substance is a quick setting dental casting material.


METRIC 1

A uniform 1 cm. distance of tissue was left exposed between the mounting blocks. All specimens were brought to room temperature and rehydrated in a normal saline bath 2 hours before testing and remained in such a state until placed in the torsion testing device. Immediately prior to testing bones from each group were randomly inspected under magnification (20X) for microfractures (Wild Heerbrugg 400 Stereomicroscope, Heerbrugg, Switzerland).

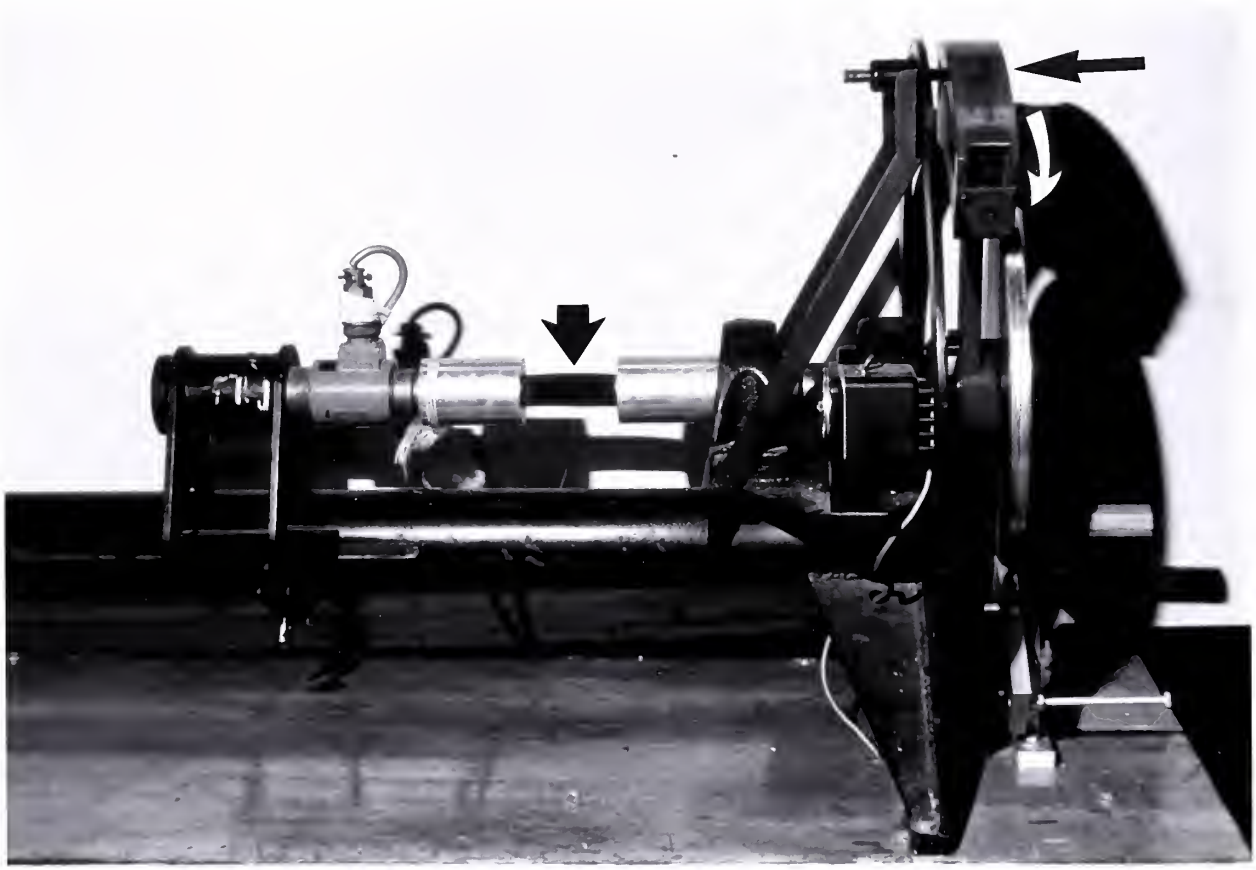
Each bone was tested to failure in torsion at a rate of 13.2 radians/sec in a torsion testing device (A.H. Burstein, Shaker Heights, Ohio) (13) (Figs. 8,9). The rate at which a bone is deformed in torsion is defined by either a strain rate or the rate of torsional deformation. Torsional deformation is easier to assess than the strain rate because of the complex geometry of a cross section of bone. A dual-beam oscilloscope (Tekitronics Type 561B) graphically recorded the load deformation curve (Fig. 10). The loading of the bone was achieved by a falling pendulum which engaged one end of the bone immediately prior to reaching resting position. Transducers measured the torsional deformation. A microswitch, triggered by the falling pendulum, activated the oscilloscope sweep just before the bone started to load. The information was stored in a personal computer (DFI) and the biomechanical parameters were calculated. The maximum torque was considered to be the point at which the femur failed. The 4 parameters assessed were maximum torque, torsional stiffness, angular deformation at failure and energy absorbed at failure.

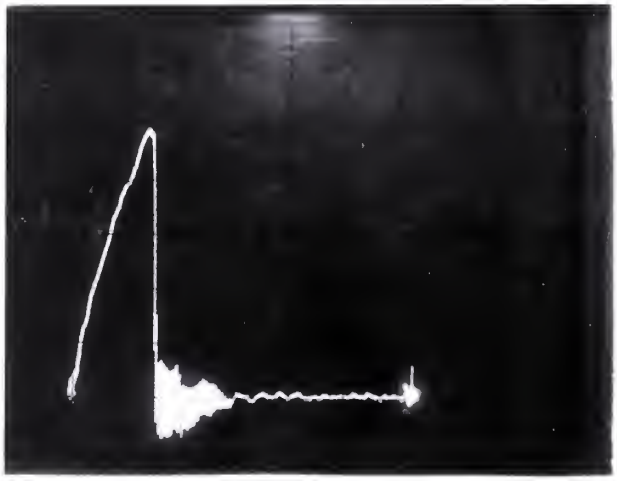
Comparisons were made within the groups utilizing the paired t-test and unnormalized data. The experimental values were also normalized against the contralateral controls as relative ratios and the means, standard deviations, and standard errors of the means calculated. Analysis of variance (ANOVA) was used to compare values between groups employing the means of the normalized ratios for each group and the respective standard deviations.

Fig. 8. Torsion testing device. The mounted femur was placed horizontally (thick black arrow) with each end locked into a torsion mount cylinder. The mount cylinder on the right was engaged by the fulcrum (thin black arrow) just prior to reaching its resting point as it swung down in an arc at a rate of13.2 radians/sec(white curved arrow).

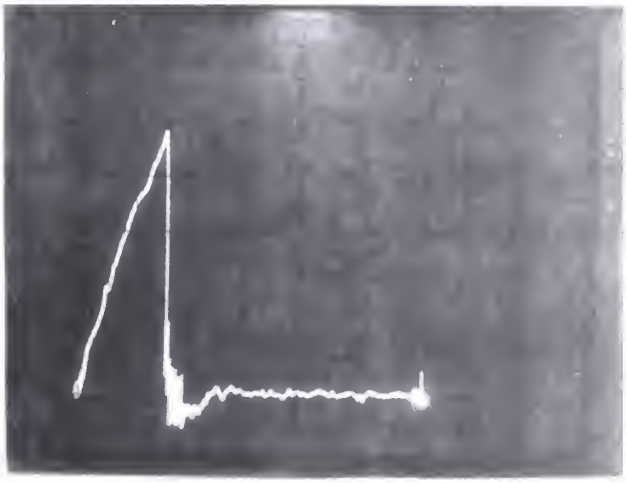
Fig. 9. Experimental set up. The dual-beam oscilloscope (arrow) graphically recorded the load deformation curve while the information was stored in the personal computer to the left of the oscilloscope. The biomechanical parameters were then calculated.

Fig.10. Typical load deformation curves for each experimental group as displayed by the oscilloscope. A) Control femur for Group 1 (irradiated), B) Experimental femur for Group 1 (irradiated), C) Experimental femur for Group 2 (freeze-dried), D) Experimental femur for Group 3 (irradiated then freeze-dried), E) Experimental femur for Group 4 (freeze-dried then irradiated), F) Control femur for group 4 (freeze-dried then irradiated).

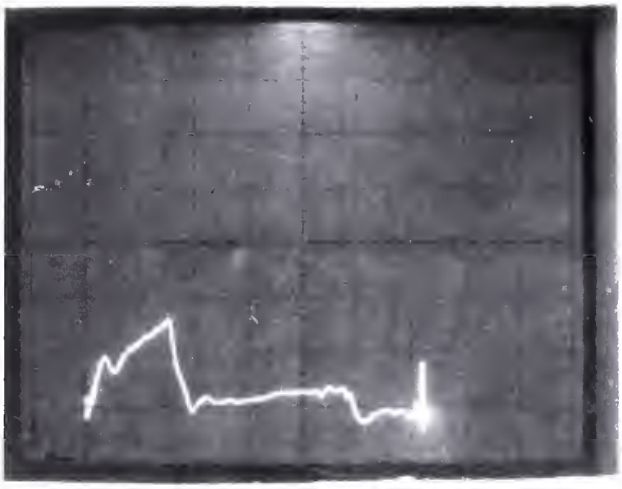




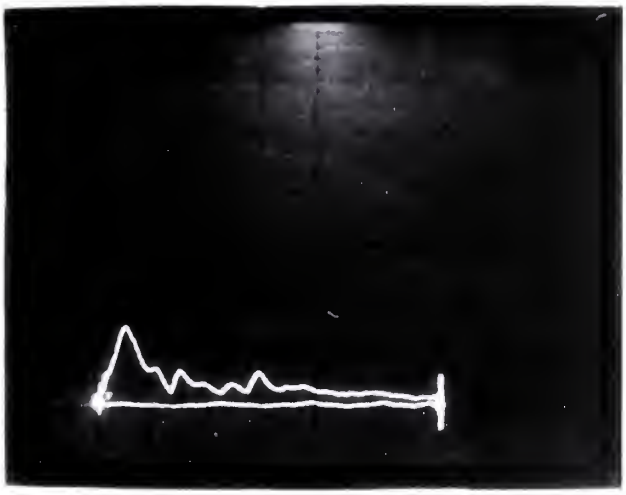
A



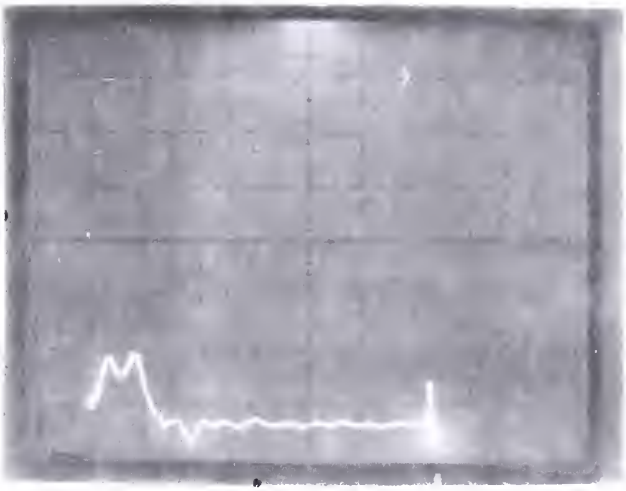
B



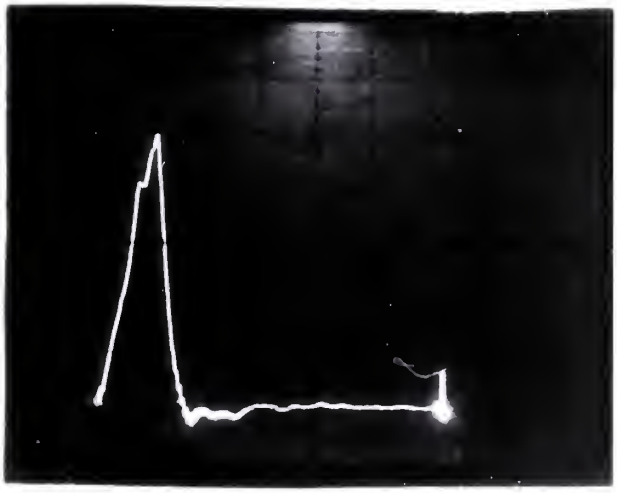
C



D



E



F

Results

Following losses to handling and processing, the respective number of pairs of femurs in each group available for testing was 9,10,7 and 7. Microfractures (Fig. 11) were observed after rehydration in nearly all (>85%) of the specimens in the three groups that underwent freeze-drying as part or all of their treatment (Table 3). Specimens that only underwent irradiation were free of microfractures.

TABLE 3. *Microfractures*

	RAD	FD	RAD/FD	FD/RAD
Control	0/9*	0/10	0/7	0/7
Expt.	0/9	9/10	6/7	7/7

*Numerator represents number of bones in a given group with at least one microfracture, denominator represents number of bones inspected.

Spiral fracture patterns were consistently observed in the bones when loaded to failure. Occasionally, fractures were noted to extend into the adjacent potted areas however the extent of this was not quantitated.

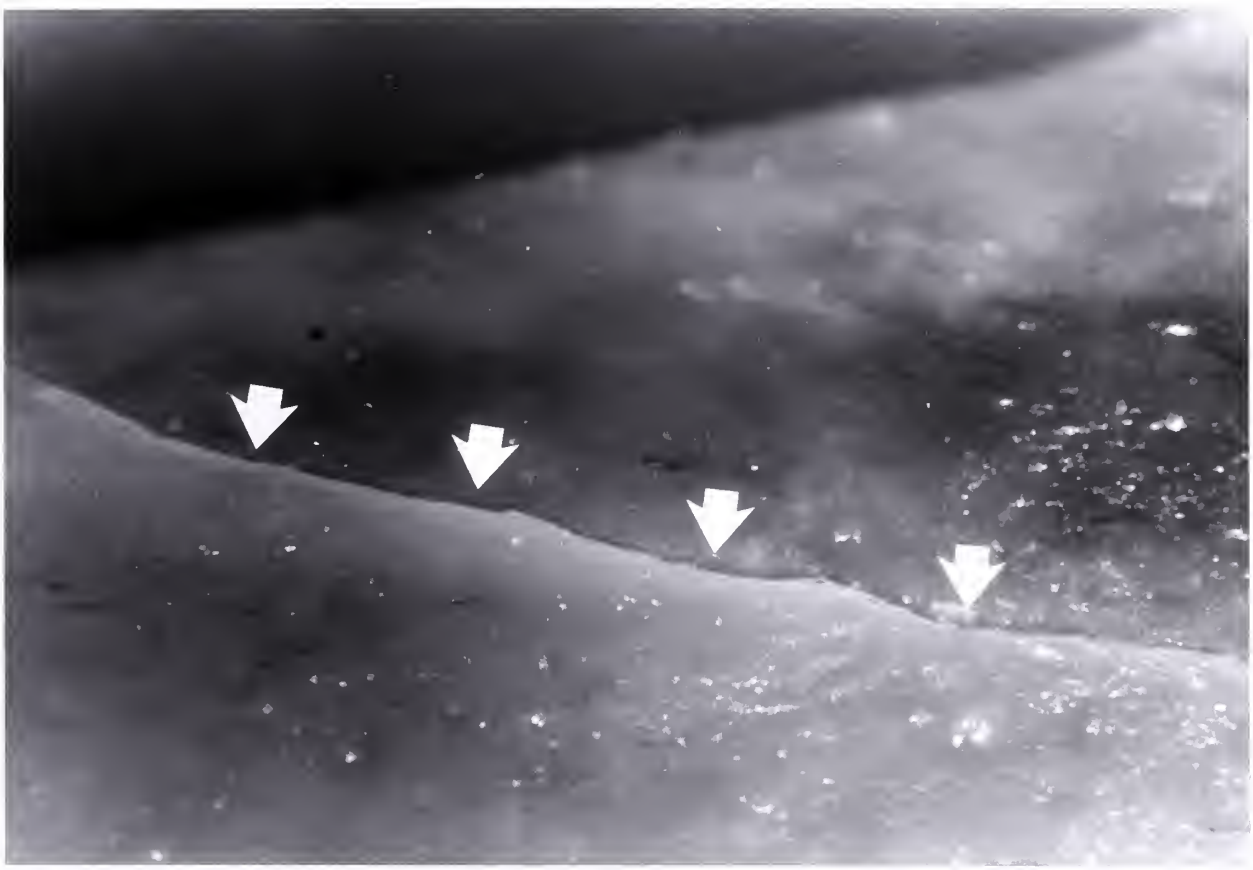
Intragroup comparisons were made using the unnormalized mean parametric results (Table 4).

TABLE 4. *Unnormalized mean parametric values ±SEM*

		RAD N=9	FD N=10	RAD/FD N=7	FD/RAD N=7
TORQUE	C	.73±.10	.51±.05	.39±.08	.50±.04
(Nm)	E	.68±.11	.14±.02*	.08±.01*	.07±.01*
STIFFNESS	C	5.5±1.8	3.7±.29	2.2±.31	3.5±.24
(Nm/rad)	E	6.2±1.3	1.1±.22*	1.2±.22*	1.0±.25*
ANGLE	C	13.±3.9	8.0±.88	9.5±1.2	8.5±1.1
(Degree)	E	6.4±1.1	9.5±.22	4.6±.69*	5.2±1.0*
ENERGY	C	.06±.02	.04±.01	.04±.01	.04±.01
(Nm)	E	.02±.00	.01±.00*	.01±.00*	.00±.00*

*P<.05, control (C) compared to experimental (E).

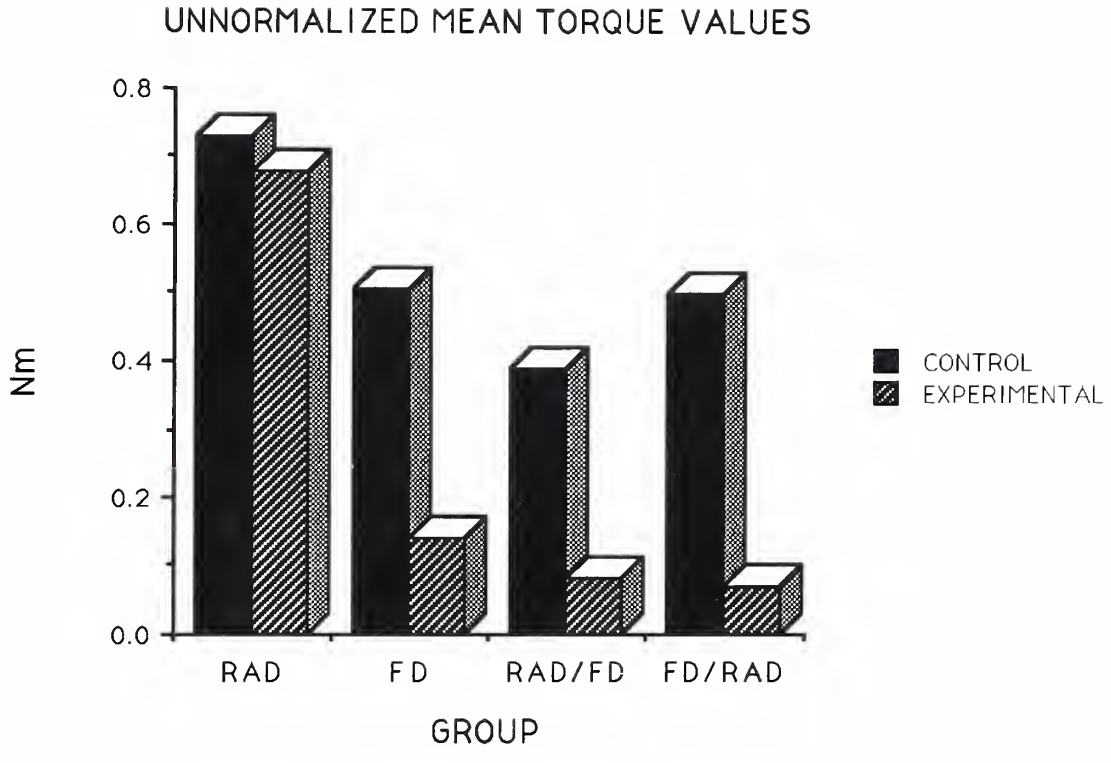
Fig. 11. Microfracture as seen through surgical microscope (magnified 20X).



Group 1 (irradiated specimens) showed no significant change with respect to their contralateral controls for any of the biomechanical parameters. Group 2 (freeze-dried) revealed a significant decrease in the torsional strength, stiffness and energy absorbed at failure ($p<.05$) although the increase in angle to failure was not significant ($p>.05$). All 4 biomechanical parameters were significantly changed in Group 3 (irradiated/freeze-dried)) and Group 4 (freeze-dried/irradiated) ($p<.05$). On the next two pages are presented the results according to graphs of the four different biomechanical parameters and their affects on each of the four groups (Figs. 12,13,14,15).

Fig.12. The unnormalized mean torque values for each experimental group and their controls, irradiated (RAD), freeze-dried (FD), irradiated then freeze-dried (RAD/FD) and freeze-dried then irradiated (FD/RAD). The differences between experimental and control mean values for each group were all statistically significant ($p<.05$) except for RAD.

Fig. 13. The unnormalized mean stiffness values for each experimental group and their controls, irradiated (RAD), freeze-dried (FD), irradiated then freeze-dried (RAD/FD) and freeze-dried then irradiated (FD/RAD). The differences between experimental and control mean values for each group were all statistically significant ($p<.05$) except for RAD.


UNNORMALIZED MEAN TORQUE VALUES
Nm
0.8
0.6
0.4
0.2
0.0
RAD
FD
RAD/FD
FD/RAD
GROUP
CONTROL
EXPERIMENTAL

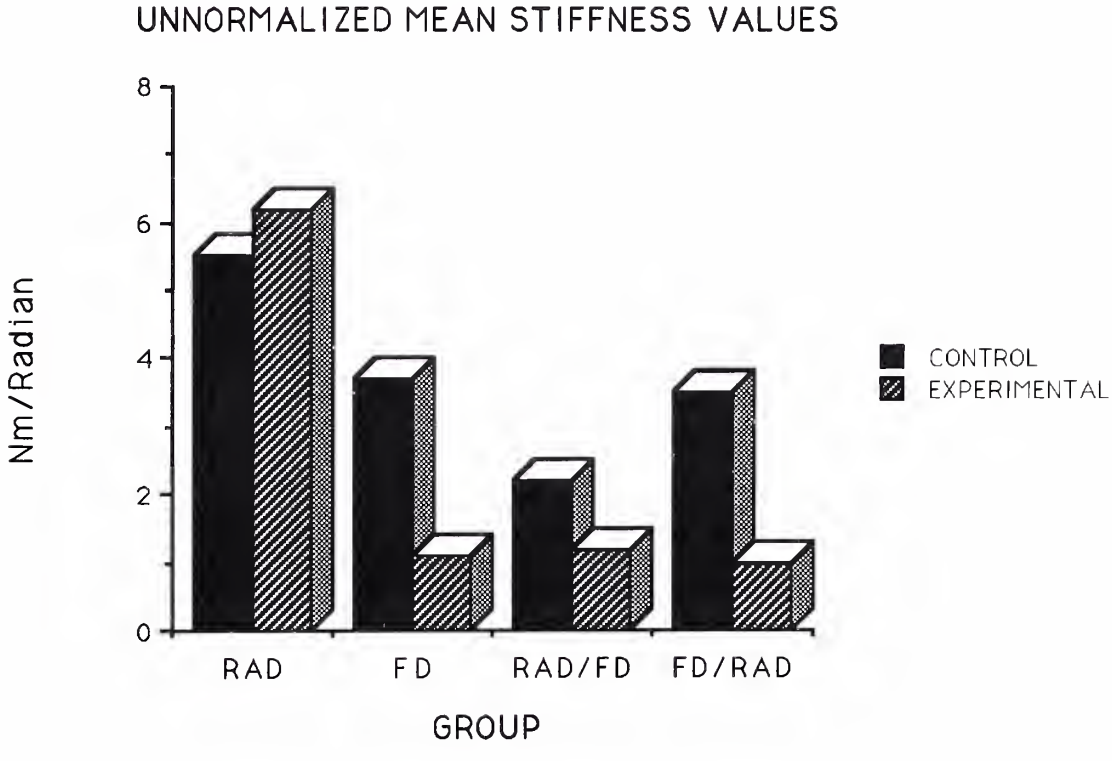
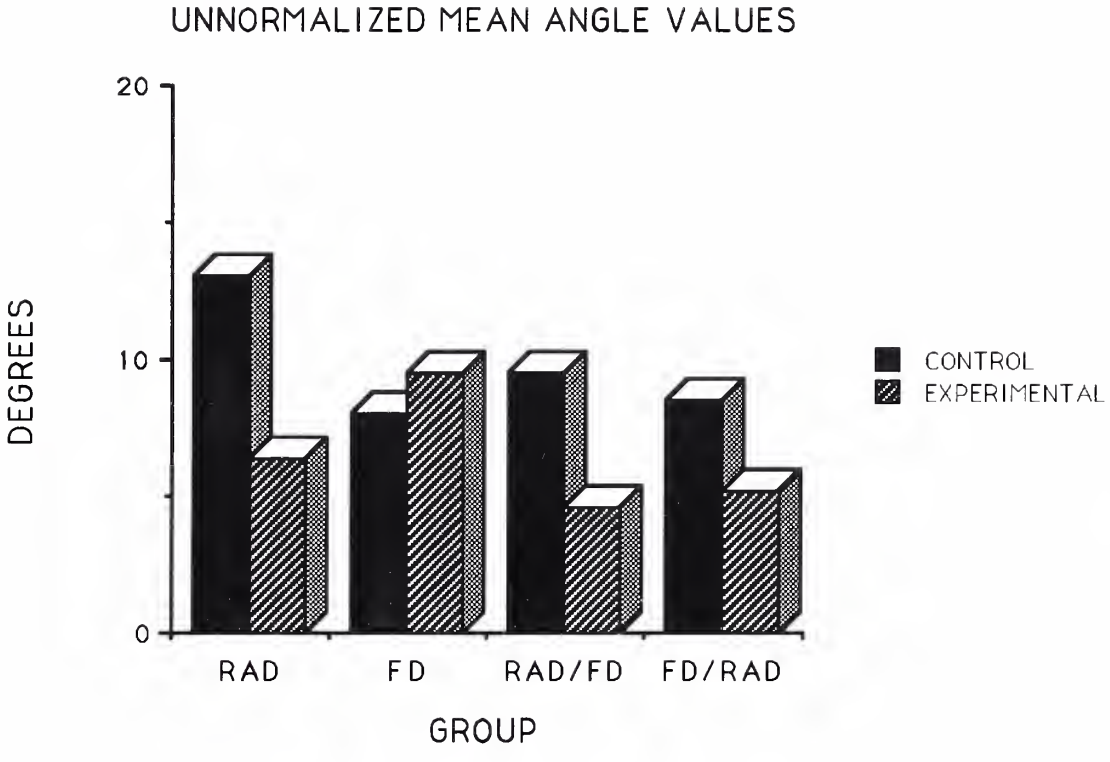
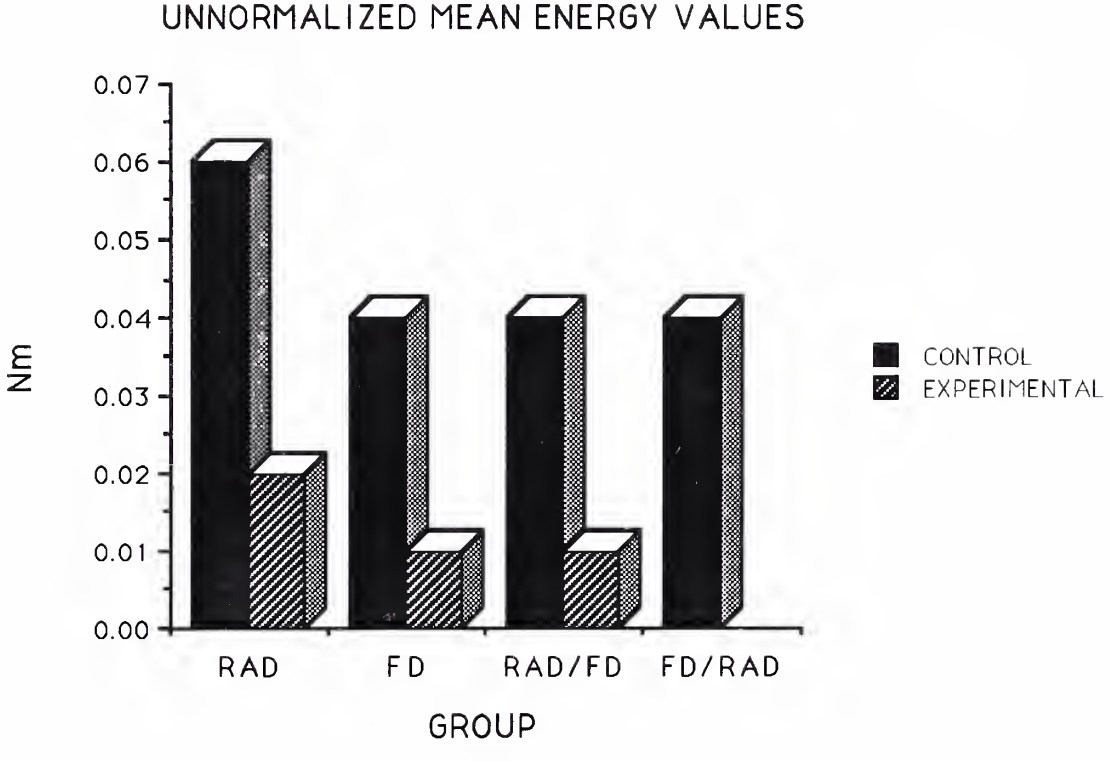

UNNORMALIZED MEAN STIFFNESS VALUES
Nm/Radian
8
6
4
2
0
RAD
FD
RAD/FD
FD/RAD
GROUP
CONTROL
EXPERIMENTAL

Fig. 14. The unnormalized mean angle values for each experimental group and their controls, irradiated (RAD), freeze-dried (FD), irradiated then freeze-dried (RAD/FD) and freeze-dried then irradiated (FD/RAD). The differences between experimental and control mean values for each group were statistically significant for RAD/FD and FD/RAD ($p<.05$) but not for RAD and FD.

Fig. 15. The unnormalized mean energy values for each experimental group and their controls, irradiated (RAD), freeze-dried (FD), irradiated then freeze-dried (RAD/FD) and freeze-dried then irradiated (FD/RAD). The differences between experimental and control mean values for each group were all statistically significant ($p<.05$) except for RAD.


UNNORMALIZED MEAN ANGLE VALUES
DEGREES
20
10
0
RAD
FD
RAD/FD
FD/RAD
GROUP
CONTROL
EXPERIMENTAL


UNNORMALIZED MEAN ENERGY VALUES
Nm
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
RAD
FD
RAD/FD
FD/RAD
GROUP
CONTROL
EXPERIMENTAL

Using the ANOVA test, the relative ratios (experimental/control) for the torques of the specimens that were freeze-dried (.32), irradiated then freeze-dried (.40) and freeze-dried then irradiated (.14) were all much lower than those only irradiated (1.0) (Fig. 16). This was statistically significant with a 95% confidence level. Those bones that were freeze-dried then irradiated appeared weaker than those either freeze-dried or irradiated then freeze-dried, however this was not statistically significant. No significant change was found between those bones that were first irradiated and then freeze-dried and those bones that were either freeze-dried or those that were freeze-dried and then irradiated.

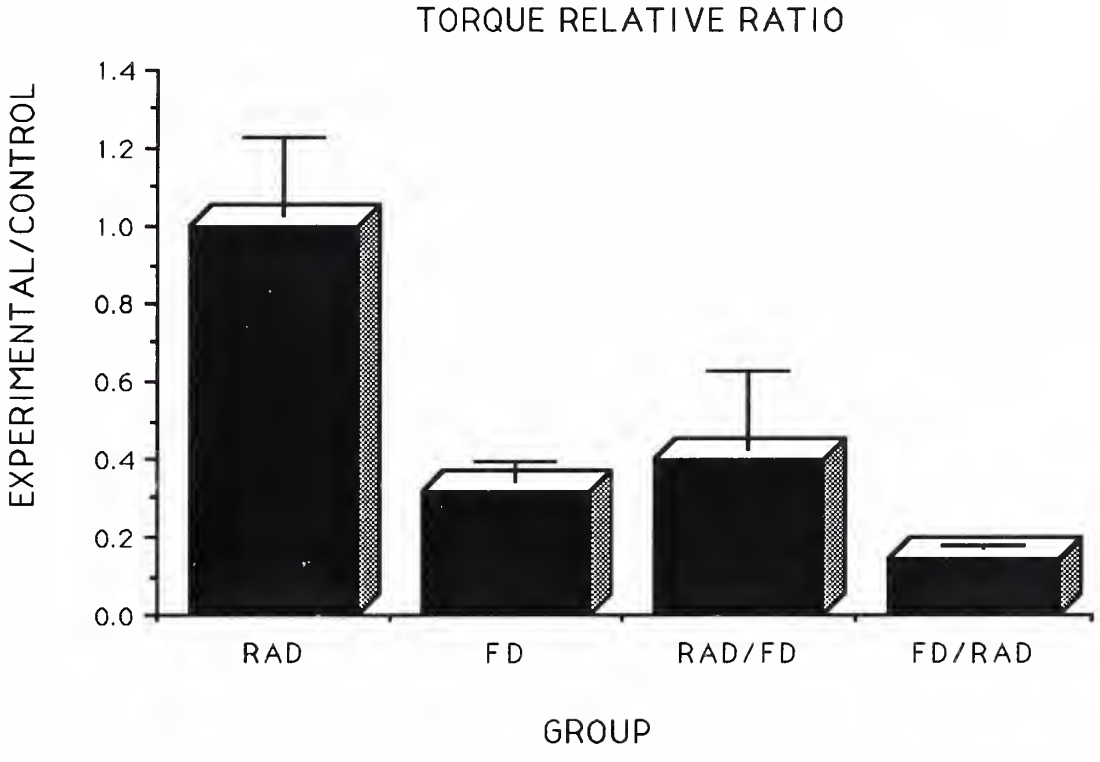


FIG.16. The torque relative ratios for the four experimental groups, irradiated (RAD), freeze-dried (FD), irradiated then freeze-dried (RAD/FD) and freeze-dried then irradiated (FD/RAD). Each experimental value was normalized against its contralateral control as a ratio. The mean of these values for each group was then calculated as represented by the bars. The SEM for each group is indicated. The RAD group was statistically significant from the other three groups ($p<.05$).

The relative ratios for the stiffness exhibited a similar pattern to that of the torque ratios with a statistically significant difference between Group 1 (irradiated =1.6) and the other 3 groups but not between these 3 latter groups (freeze-dried=.29, irradiated/freeze-dried=.59 and freeze-dried/irradiated=.29) (Fig. 17).

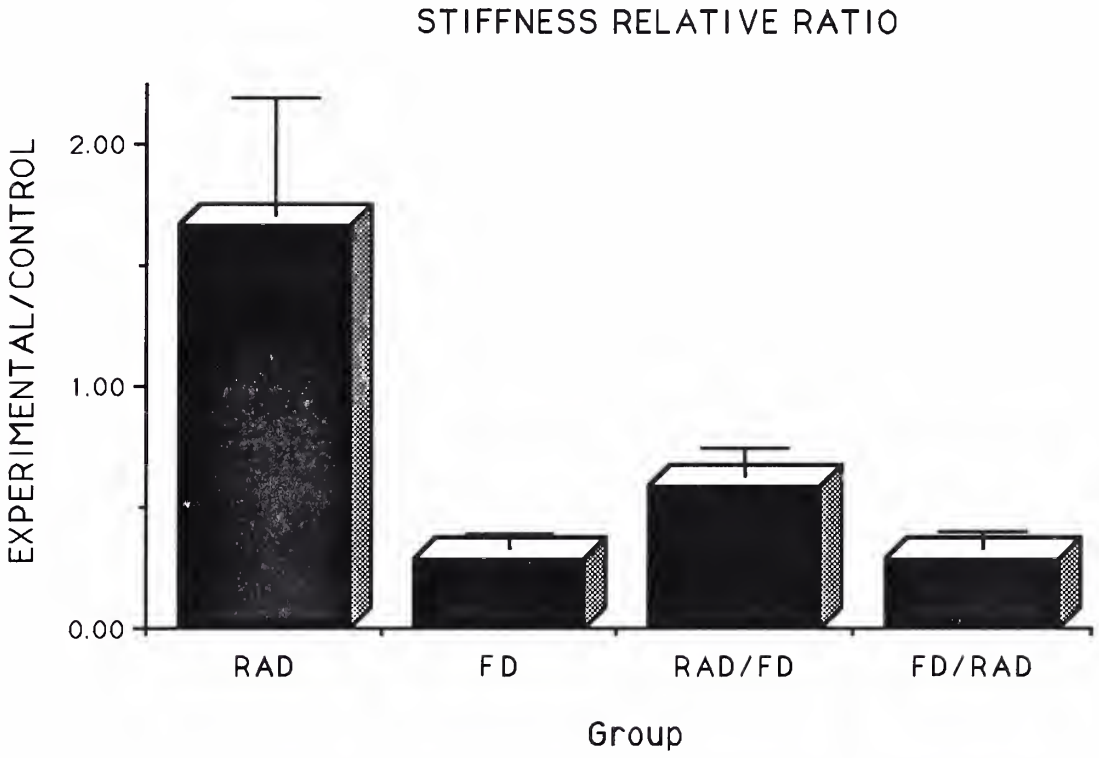


FIG.17. The stiffness relative ratios for the four experimental groups, irradiated (RAD), freeze-dried (FD), irradiated then freeze-dried (RAD/FD) and freeze-dried then irradiated (FD/RAD). Each experimental value was normalized against its contralateral control as a ratio. The mean of these values for each group was then calculated as represented by the bars. The SEM for each group is indicated. The RAD group was statistically significant from the other three groups ($p<.05$).

Discussion

These results confirm that freeze-drying significantly decreases the torsional strength, stiffness and energy absorbed to failure as compared to control in the long bone. When the specimen is irradiated prior to freeze-drying there appears to be no additional effect with respect to torsional strength. In this study there was actually a minimal increase in torsional strength as compared to specimens that were only freeze-dried. If however the bone was irradiated after freeze-drying there appeared to be a noticeable although statistically insignificant trend toward an additional decrease in the torsional strength of long bone. The stiffness of the bone did not appear to be further decreased by subsequent irradiation after freeze-drying. An increase in stiffness was exhibited in bones irradiated prior to freeze-drying, a trend also seen with respect to torsion.

The paired t-test was chosen to analyze the unnormalized data as the specimens were matched by having the contralateral femurs serve as control. The hypothesis that the experimental groups differed from each other by more than random chance, with a 95% confidence level, was addressed by analysis of variance. Note that while it is called an analysis of variance its purpose is to actually assess differences between the group means.

A Mechanism Is Proposed

In postulating a mechanism to account for these findings one must consider the presence of microfractures in only those bones that were subjected to freeze-drying as part or all of their treatment. This supports the previous finding that freeze-drying appears to be particularly detrimental to the torsional strength of long bone (57). When water is frozen it expands secondary to the crystaline lattice configuration it acquires and thereby increases the internal

stress within the osseous tissue. Freeze-drying of bone results in sublimation of ice directly into the gaseous state bypassing the liquid state. Such a process may result in the microfracturing observed.

The heating of the bone to 100°C during the gravimetric assay phase of the freeze-drying protocol may significantly damage the collagen macromolecule. The shrinkage temperature (Ts) is the index for assessing the thermostability of intact collagen fibers. At a given temperature the collagen fiber shortens markedly from loss of the helical structure of tropocollagen (the subunit of the collagen macromolecule). The mammalian Ts is 65°C (Calf skin) (70). Interestingly, the thermal stability for a given species is correlated with the content of imino acids (proline and hydroxyproline) in collagen as well as core body temperature. While the Ts of rat bone collagen may be marginally different from that of calf skin, a temperature of 100°C is well above the thermostability point.

The effects of irradiation on peptide bonds and the formation of crosslinks in collagen have been studied (17,22,46,67). Gamma irradiation at 1 Mrad is able to cleave a significant number of collagen molecules (17). What percentage of this protein damaging is a direct result of the irradiation as opposed to indirect damage secondary to hydroxyl-free radical production from cleaved water is not known. However, dosage correlates directly with the amount of damage and therefore at doses above 3.0 Mrads a large portion of the bone collagen is significantly altered. Interestingly our study revealed no significant biomechanical difference in those specimens undergoing 3.0 Mrad irradiation as compared to control.

Gamma rays, which are high-energy photons, lose energy by Compton scattering or pair (positron, electron) production. This particular form of energy

release may affect the hydroxyapatite mineral portion of bone as well as the organic material.

The percentage water content of the bone and the relative type of water, free or bound, may be a factor in the degree to which the irradiation induces chemical changes within the osseous tissue. During lyophilization the free water is driven off first with the residual water in the bound state. Therefore at a given irradiation dosage hydroxyl-free radical production will occur to bound water in freeze-dried tissue as opposed to free and bound water in fresh or frozen bone. This may result in greater damage to the collagen macromolecule in tissue that has been previously freeze-dried.

Megadose irradiation has been shown to impart a modest rise in temperature in the tissue undergoing sterilization (12). An increase of 10°C was noted with a dosage of 2.5 Mrads delivered over 10 minutes. Such heating may reduce the effect of freeze-drying by decreasing the relative amount of water in the tissue to be frozen and subsequently lyophilized. This might impart minimal protection to the detrimental effect of freeze-drying on the bone. Since the irradiation itself has its own additional deleterious effects, perhaps as an inverse function of water content by the above mentioned mechanisms, a sequential dependency may result. If the tissue is first freeze-dried then irradiation would damage the proteins by affecting the macromolecules directly and by creating hydroxyl-free radicals in water bound to the collagen without the "buffer" of free water to absorb the energy.

To investigate this hypothesis further investigation into the biochemistry of lyophilized versus hydrated collagen's ability to be cleaved and crosslinked by gamma irradiation is necessary. Protein electrophoresis would demonstrate different collagen banding patterns depending upon the order of treatment. Dessicated collagen that is subsequently irradiated should reveal smaller

fragments than bone treated in the reverse order because of the increase degree of cleavage either from irradiation directly or from greater hydroxyl-free radical production in the bound water.

In addition, one might quantitate the extent of microfractures observed in terms of the number of microfractures per bone per group. Microfractures would be more prevalent in bone with a greater initial water content prior to lyophilization than in bone that is freeze-dried prior to irradiation.

To assess the amount of moisture driven off by megadose irradiation the bones subjected to such treatment should undergo gravimetric assay.

Critique of Design

Selection of the irradiation dosage used in this study reflected current standards employed by tissue banks and the International Atomic Energy Agency (2.5 Mrads) (7,32,45,69,71) for sterilization of bone. Because of the concern for HIV transmission increasing the dosage of irradiation has been suggested although the definitive dose required to inactivate HIV in infected bone is currently unknown. Studies have demonstrated as much as a 10% decrease in torsional strength at 3.0 Mrads and up to a 35% decrease at 6.0 Mrads (34). Our intent was to utilize a practical dose with regards to preservation of biomechanical strength and yet to maximize this dose to effect specimen sterility. In this study, 3.0 Mrads alone did not have a noticeable effect on the biomechanical properties of bone. It is unclear as to whether this additional 0.5 Mrads will provide the level of irradiation necessary to inactivate HIV.

The freeze-dried femurs were rehydrated in a normal saline bath for two hours prior to testing. This has been shown to be sufficient time to return the elastic modulus of bone to normal . Nevertheless the plastic modulus has been noted to remain elevated in lyophilized tissue for over 24 hours (10,11). In a test

such as this, however, it is the elastic modulus which is the major determinant in evaluating bone strength

Relatively large standard errors were observed. This may have been a result of the use of whole bone specimens in torsion testing (50). It was decided not to use machined specimens as a matter of the clinical relevance with regard to massive osseous and osteochondral transplantation as well as concern for the loss of the postulated microfracturing effect with machined samples. A greater number of specimens may have limited the effect of the inherent variability of whole bone on the differences found between treatment groups.

To determine the approximate number of specimens per group to substantiate a statistically significant difference in torsional strength between groups that were either irradiated and then freeze-dried or visa-versa a power calculation was performed employing the data from groups 3 and 4. The specifications of the formula below require that the standard deviations for the groups be equal. This was not the case and therefore the larger value was employed to ensure an adequate sample size.

$$n = \left[\left(z_\alpha - z_\beta \right) \sigma / \mu_3 - \mu_4 \right]^2$$

where n is the predicted sample size, $Z_{\alpha,\beta}$ are the upper and lower percentage points, σ is the population standard deviation and $\mu_{3,4}$ are the means of the two groups. A two tailed test is the standard and therefore at the 95 percent level of confidence to reject the null hypothesis $Z_\alpha = 1.96$. If $\mu_3 = .40$ and $\mu_4 = .14$ a 5 percent risk of failing to substantiate that the latter is statistically lower would mean a $Z_\beta = -1.65$. Thus,

$$n = \left[(1.96+1.65).52 / .40 - .14 \right]^2 = 52.1$$

or 52 experimental femurs per group. This is a rough approximation based upon the experimental standard deviation of group 3.

Relatively large ranges were noted in the control values for the four parameters (torque= .4 -.7, stiffness= 2.2 - 5.5, angle= 8-13, energy= .04 -.06) while the animal weight coefficient of variance was less than 2%. This is difficult to explain. The irradiated group, Group 1, whose control values are the greatest relative to the other three experimental groups, were tested first, several weeks before the others and thus were stored at -20°C for a shorter period of time. However, as previously discussed freezing to as low as -70°C for as long as two weeks has been shown to have no deleterious effect on the torsional strength of bone (34, 56).

The mean of the stiffness ratios, as depicted in figure 17, demonstrates that for the irradiated group the ratio is 1.6. This results from two outlyers raising their ratio values to 4. Without these two figures the mean of ratios is 1.0.

It should be noted that torsional strength of bone appears to be effected by freeze-drying while compression related parameters are not influenced (57). The clinical implications of this discrepancy, while limited, would dictate that during reconstruction adequate support by internal or external fixation must be provided to the freeze-dried graft in order to shield the load bearing bone from torsional forces in particular. During the healing stage, the bone may not need to be shielded from normal physiologic compression loads. This investigation suggests that such allografted bone may be irradiated with up to 3.0 Mrads prior to freeze-drying without an additional decrease in the torsional strength of the graft while irradiating with the same dose afterwards may increase the risk of mechanical failure of the graft.

Limits of Application

The use of rat long bones limits the clinical significance of this study. The cortex of rat bone differs from that found in humans with respect to osteal architecture in that the extent of the Haversian system is not so well developed. Nevertheless it has been shown that 300g rats demonstrate comparable dynamic histologic architecture to adult human trabecular bone (75). In considering clinical biomechanics, the size of the rat bone (Fig. 7) is substantially smaller than any allograft material that would be utilized in humans. Microfractures similar to those observed in the rat femurs might be relatively less detrimental to the structure of a larger graft assuming that the size of the microfractures does not increase with the size of the graft. However this is controversial.

The benefits of using the rat include its common use as an experimental model in assessing bone graft biology, its relative homogeneity and its availability in adequate numbers to permit intergroup comparisons. Access to sufficient numbers of paired human cadaveric long bones is limited, especially if also matched for age and sex.

The results of this study apply only to the initial properties of the bone allograft at the time of implantation. Once the graft is placed within the host site it is subjected to the continuous biological processes of revascularization, incorporation, and remodelling. In the case of allogeneic tissue, these physiologic events are in part a function of the histocompatibility differences and the ensuing immune responses.

Conclusion

The order in which bone is subjected to freeze-drying and irradiation *in-vitro* may alter its biomechanical properties. Freeze-drying preceded by irradiation does not lead to a decrease in the biomechanical properties of bone relative to freeze-drying alone. If however the bone is first freeze-dried then irradiated there tends to be a greater loss of torsional strength in the bone although this failed to reach statistical significance. The stiffness of the bone did not appear to be further effected by irradiation after freeze-drying. Given the results of this study approximately 50 samples per experimental group would be necessary to establish statistical significance. This investigation suggests that the order of the treatment of bone and not simply the treatments themselves have a noticeable effect on the biomechanical properties of osseous tissue. However, further studies, at the molecular as well as biomechanical levels, are required to determine if such a sequential dependency is of clinical consequence.

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Appendix

Group 1

		Torque	Angle	Stiffness	Energy
Raw Data					
RAD-C	1	0.469	9.616	2.795	0.041
	2	0.715	11.024	3.716	0.073
	3	0.613	11.628	3.023	0.072
	4	0.642	43.010	0.856	0.198
	5	0.606	12.634	2.748	0.073
	6	1.293	5.069	14.618	0.018
	7	0.528	14.605	2.072	0.059
	8	1.204	4.466	15.450	0.022
	9	0.488	6.840	4.088	0.020
RAD-E	1	0.457	13.438	1.947	0.045
	2	0.714	6.277	6.516	0.014
	3	1.262	5.834	12.394	0.021
	4	0.001	0.201	0.357	0.000
	5	0.650	7.041	5.288	0.028
	6	0.958	5.472	10.036	0.018
	7	0.731	5.069	8.266	0.015
	8	0.758	6.679	6.500	0.035
	9	0.562	7.484	4.303	0.035
Paired t-test Values		0.41 NS	1.53 NS	0.38 NS	1.84 NS

NS= not significant (p value > .05)

S= significant (p value $\leq$ .05)

Group 2

		Torque	Angle	Stiffness	Energy
Raw Data					
FD-C	1	0.493	10.219	2.764	0.042
	2	0.445	9.214	2.769	0.031
	3	0.651	9.817	3.800	0.058
	4	0.470	5.271	5.114	0.015
	5	0.157	3.018	2.977	0.004
	6	0.689	8.087	4.879	0.050
	7	0.613	12.231	2.874	0.058
	8	0.478	5.874	4.662	0.015
	9	0.365	6.880	3.040	0.022
	10	0.700	9.857	4.069	0.060
FD-E	1	0.094	9.053	0.592	0.010
	2	0.223	13.438	0.949	0.031
	3	0.138	16.818	0.470	0.025
	4	0.135	10.139	0.766	0.013
	5	0.104	3.018	1.977	0.003
	6	0.194	4.627	2.400	0.006
	7	0.110	12.030	0.523	0.015
	8	0.099	6.920	0.821	0.007
	9	0.124	11.869	0.599	0.018
	10	0.209	7.403	1.616	0.009
Paired t-test Values		7.442 S	1.320 NS	8.488 S	2.900 S

NS= not significant (p value > .05)

S= significant (p value $\leq$ .05)

Group 3

		Torque	Angle	Stiffness	Energy
Raw Data					
RAD/FD-C	1	0.715	12.835	3.192	0.082
	2	0.081	2.776	1.683	0.000
	3	0.444	9.455	2.691	0.034
	4	0.114	9.214	0.710	0.007
	5	0.463	10.300	2.575	0.039
	6	0.460	10.702	2.465	0.043
	7	0.434	11.427	2.176	0.039
RAD/FD-E	1	0.072	3.661	1.129	0.003
	2	0.127	4.466	1.634	0.005
	3	0.093	2.454	2.167	0.002
	4	0.051	4.627	0.637	0.003
	5	0.062	4.466	0.789	0.003
	6	0.085	8.409	0.577	0.088
	7	0.090	4.225	1.225	0.003
Paired t-test Values		3.523 S	3.580 S	12.200 S	3.0196 S

NS= not significant (p value > .05)

S= significant (p value $\leq$ .05)

Group 4

		Torque	Angle	Stiffness	Energy
Raw Data					
FD/RAD-C	1	0.405	6.320	3.680	0.038
	2	0.629	9.012	3.996	0.051
	3	0.596	9.817	3.478	0.051
	4	0.566	12.030	2.695	0.055
	5	0.537	11.748	2.619	0.050
	6	0.314	4.225	4.253	0.009
	7	0.453	6.679	3.885	0.023
FD/RAD-E	1	0.062	1.650	2.135	0.000
	2	0.060	6.639	0.520	0.004
	3	0.070	2.857	1.396	0.001
	4	0.094	8.811	0.608	0.007
	5	0.074	7.685	0.552	0.005
	6	0.045	6.035	0.423	0.002
	7	0.083	3.018	1.572	0.002
Paired t-test Values		10.676 S	3.268 S	7.858 S	6.251 S

NS= not significant (p value > .05)

S= significant (p value $\leq$ .05)

ANOVA Test for Torsional Strength

Group	Mean	S.D.	N
RAD	1.0	.56	9
FD	.32	.15	10
RAD/FD	.40	.52	7
FD/RAD	.14	.03	7

ANOVA Summary Table

Source of Variations	DF	Sum of Squares	Mean	F
Between Groups	3	3.54	1.18	7.89
Within Groups	29	4.33	.15	
Total	32	7.88		

F (3, 29, .95)= 2.92

Group	vs	Group	S/NS
RAD		FD	S
RAD		RAD/FD	S
RAD		FD/RAD	S
FD		RAD/FD	NS
FD		FD/RAD	NS
RAD/FD		FD/RAD	NS

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